ORIGINAL ARTICLE

# The *MDM2* promoter polymorphism SNP309T→G and the risk of uterine leiomyosarcoma, colorectal cancer, and squamous cell carcinoma of the head and neck

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**Background:** MDM2 acts as a principal regulator of the tumour suppressor p53 by targeting its destruction through the ubiquitin pathway. A polymorphism in the MDM2 promoter (SNP309) was recently identified. SNP309 was shown to result, via Sp1, in higher levels of MDM2 RNA and protein, and subsequent attenuation of the p53 pathway. Furthermore, SNP309 was proposed to be associated with accelerated soft tissue sarcoma formation in both hereditary (Li-Fraumeni) and sporadic cases in humans.
**Methods:** We evaluated the possible contribution of SNP309 to three tumour types known to be linked with the MDM2/p53 pathway, using genomic sequencing or restriction fragment length polymorphism as screening methods. Three separate Finnish tumour materials (population based sets of 68 patients with early onset uterine leiomyosarcomas and 1042 patients with colorectal cancer, and a series of 162 patients with squamous cell carcinoma of the head and neck) and a set of 185 healthy Finnish controls were analysed for SNP309.
**Results:** Frequencies of SNP309 were similar in all four cohorts. In the colorectal cancer series, SNP309 was somewhat more frequent in women and in patients with microsatellite stable tumours. Female SNP309 carriers were diagnosed with colorectal cancer approximately 2.7 years earlier than those carrying the wild type gene. However, no statistically significant association of SNP309 with patients' age at disease onset or to any other clinicopathological parameter was found in these three tumour materials.
**Conclusion:** SNP309 had no significant contribution to tumour formation in our materials. Possible associations of SNP309 with microsatellite stable colorectal cancer and with earlier disease onset in female carriers need to be examined in subsequent studies.

The tumour suppressor p53 is a principal mediator of a number of cellular functions, including growth arrest, senescence, and apoptosis in response to DNA damage.[1] As such, *p53* is the most frequently mutated gene in human tumours; alterations in the *p53* gene are found in approximately 50% of all cancers.[2] MDM2 acts as a key regulator of p53 through an autoregulatory feedback loop. MDM2 is capable of binding p53 and regulating its ability as a transcriptional activator. MDM2 is also involved in the nuclear export of p53, and serves as an E3 ubiquitin ligase that promotes p53 destruction.[1 3] Additionally, MDM2 seems to interact with other cellular proteins that are important in cell cycle control, including pRb, E2F/DP1, and p19ARF, although some of these functions are less well characterised.[4] Amplification or overexpression of MDM2 commonly occurs in human tumours. Tumour types displaying overexpression of MDM2 include soft tissue sarcomas, osteosarcomas, rhabdomyosarcomas, oral squamous cell, colorectal and breast carcinomas, and malignant melanomas.[3 5–7] Furthermore, MDM2 overexpression has been linked with the clinical behaviour of the tumours.[6]

Recently, a single nucleotide polymorphism in the MDM2 promoter, SNP309T→G (SNP309), was identified,[8] and shown to result in overexpression of MDM2 RNA and protein and subsequent attenuation of the p53 pathway. It was further demonstrated that the heightened level of MDM2 was mediated by and dependent on transcription factor Sp1. Consequently, it was hypothesised that increased levels of MDM2 resulting from SNP309 could affect the p53 pathway in patients with germline mutations in one p53 allele (Li-Fraumeni syndrome) and have an impact on the tumour susceptibility of these individuals. To test this hypothesis, Bond *et al* studied a cohort of 88 patients with Li-Fraumeni syndrome, and found an association of the occurrence of SNP309 with earlier tumour onset, as well as with a phenotype of multiple primary cancers. In addition, in a material of 105 sporadic soft tissue sarcomas, of which 16 were leiomyosarcomas, SNP309 was found to be associated with an accelerated tumour formation.[8]

We report on our evaluation of SNP309 and its implication for disease onset in a population based set of early onset uterine leiomyosarcoma cases, and our analysis of the possible contribution of SNP309 to two other tumour types, colorectal cancer (CRC, 1042 cases) and squamous cell carcinoma of the head and neck (HNSCC, 162 cases), which are linked with the p53 pathway. Healthy blood donors served as controls.

## METHODS

### Patients

Samples from population based series of patients with early onset uterine leiomyosarcoma were analysed for SNP309. This unique material consists of tumour specimens from 68 Finnish patients diagnosed with histologically verified uterine leiomyosarcomas at $\leqslant$45 years of age. The paraffin embedded samples for this material were collected from cases

**Abbreviations:** CRC, colorectal cancer; HNSCC, squamous cell carcinoma of the head and neck; RFLP, restriction fragment length polymorphism

**Table 1** Frequencies of *MDM2* SNP309 in controls and cancer patients

| Cohorts and subgroups | No. of individuals | No. of individuals according to SNP309 genotype (%) | | | Pearson $\chi^{2*}$ |
|---|---|---|---|---|---|
| | | Wild type | Heterozygote | Homozygote | p value |
| Healthy controls | 185 | 56 (31) | 98 (53) | 31 (16) | |
| EO leiomyosarcomas | 68 | 21 (31) | 36 (53) | 11 (16) | 0.99† |
| Age <41 years | 33 | 9 (27) | 20 (61) | 4 (12) | 0.44 |
| Age >41 years | 35 | 12 (34) | 16 (46) | 7 (20) | |
| Familial cancer | 38 | 11 (29) | 19 (50) | 8 (21) | 0.47 |
| No familial cancer | 30 | 10 (33) | 17 (57) | 3 (10) | |
| Local tumour | 49 | 14 (29) | 28 (57) | 7 (14) | 0.21 |
| Diffused tumour | 5 | 2 (40) | 1 (20) | 2 (40) | |
| CRC | 969 | 334 (34) | 465 (48) | 170 (18) | 0.44† |
| Microsatellite stable | 849 | 294 (34.5) | 397 (47) | 158 (18.5) | 0.04 |
| Microsatellite unstable | 120 | 40 (33) | 68 (57) | 12 (10) | |
| Female | 481 | 147 (31) | 239 (50) | 95 (20) | 0.02 |
| Male | 488 | 187 (38) | 226 (46) | 75 (15) | |
| Multiple cancer | 124 | 42 (34) | 52 (42) | 30 (24) | 0.10 |
| A single CRC, no other Malignancies | 845 | 292 (34.5) | 413 (49) | 140 (16.5) | |
| Dukes A-B | 572 | 185 (32) | 282 (49) | 105 (18) | 0.24 |
| Dukes C-D | 391 | 147 (38) | 180 (46) | 64 (16) | |
| Grade 1 | 192 | 69 (36) | 97 (50.5) | 26 (13.5) | 0.40 |
| Grade 2 | 677 | 225 (33) | 326 (48) | 126 (19) | |
| Grade 3–4 | 80 | 32 (40) | 34 (42.5) | 14 (17.5) | |
| Familial CRC | 835 | 282 (34) | 401 (48) | 152 (18) | 0.31 |
| No familial CRC | 134 | 52 (39) | 64 (48) | 18 (13) | |
| Familial cancer | 436 | 146 (33) | 209 (48) | 81 (19) | 0.70 |
| No famililal cancer | 533 | 188 (35) | 256 (48) | 89 (17) | |
| SCCHN | 157 | 58 (37) | 75 (48) | 24 (15) | 0.47† |
| Age ⩽61 years | 84 | 32 (38) | 42 (50) | 10 (12) | 0.45 |
| Age >61 years | 73 | 26 (36) | 33 (45) | 14 (19) | |
| Recurrent SCCHN | 122 | 44 (36) | 60 (49) | 18 (15) | 0.88 |
| No recurrence | 32 | 13 (41) | 15 (47) | 4 (12) | |
| T1 tumours | 68 | 28 (41) | 31 (46) | 9 (13) | 0.6 |
| T⩾2 tumours | 89 | 30 (34) | 44 (49) | 15 (17) | |
| N0 tumours | 108 | 43 (40) | 46 (42.5) | 19 (17.5) | 0.14 |
| N+ tumours | 49 | 15 (31) | 29 (49) | 5 (10) | |

*Subgroup versus subgroup; †group versus healthy controls. EO, early onset.

diagnosed in Finland between the years 1984 and 2002 based on the Finnish cancer registry data. During this time, 80 uterine leiomyosarcomas had been diagnosed in the specified age group, thus our material represents 85% of the diagnosed cases in Finland. Twelve cases could not be analysed because no paraffin embedded tissue was available.

Population based material from 1042 Finnish colorectal cancer patients was screened for MDM2 SNP309. This well documented material has been described in more detail in previous works.[9 10]

The HNSCC material (n = 162) was from patients with HNSCC who were treated at the Department of Otorhinolaryngology Head and Neck Surgery at Helsinki University Central Hospital between 1997 and 2003 and who had given their blood sample to the tumour bank. Patients' median age at diagnosis was 62 years (range 26–89 years). The most common tumour sites were larynx (61/162), tongue (34/162), tonsils (24/162) hypopharynx (8/162), and base of the mouth (8/162).

In total, 185 healthy blood donors served as controls in the study. DNA extractions were performed using standard methods.

### Sequencing

Genotyping of the uterine leiomyosarcoma cases and the healthy controls was performed by genomic sequencing. PCR primers used in the reactions were as follows: 5′-GTTTTG TTGGACTGGGGCTA-3′ and 5′-CTGCGATCATCCGGACCT-3′. PCR was performed in 25 ul volumes containing 50 ng genomic DNA, 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 200 µmol/l each dNTP (Finnzymes, Espoo, Finland) 0.25 µmol/l both primers, and 1.25 U Ampli*Taq* Gold DNA polymerase. PCR products were purified using ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH, USA), and sequenced using AB 3100 and 3730 BD3.1 sequencing chemistry and 5.1 sequencing analysis software (Applied Biosystems).

### Restriction fragment length polymorphism

An RFLP method was used in the genotyping of the CRC and HNSCC samples. PCR was performed as described by Bond *et al*.[8] Digestion was performed in 50 µl reaction volume using *Msp*A1I restriction endonuclease (New England Biolabs, Beverly, MA, USA) according to manufacturer's instructions. Restriction fragments were visualised in 4% agarose gel electrophoresis.

### Statistical methods

Possible statistical differences in continuous variables were tested by one way analysis of variance (SPSS version 12.0 for Windows) and in dichotomous variables by Pearson's $\chi^2$ test. A p value $<0.05$ was considered significant. In the CRC material, the effect of multiple testing was corrected using Bonferroni adjustment, and $p<0.005$ was considered significant.

## RESULTS

### Healthy controls

In a cohort of 185 healthy controls, 56 individuals (30%) were wild type (TT), 98 (53%) heterozygous (TG), and 31(17%) homozygous (GG) for SNP309 (table 1).

**Table 2** Mean ages at diagnosis according to SNP309 status in cancer cohorts

| Cohorts and subgroups | No. of individuals | Mean age at diagnosis (years) | | Pearson $\chi^{2*}$ |
|---|---|---|---|---|
| | | WT | SNP309 | |
| Leiomyosarcoma | 68 | 40.9 (n = 21) | 39.3 (n = 47) | 0.24 |
| CRC | 969 | 68.1 (n = 334) | 67.0 (n = 635) | 0.17 |
| Female | 481 | 70.5 (n = 147) | 67.8 (n = 334) | 0.026 |
| Male | 488 | 66.2 (n = 187) | 66.1 (n = 301) | 0.92 |
| MSS CRC* | | | | |
| Female | 417 | 70.8 (n = 123) | 67.31 (n = 294) | 0.007 |
| Male | 432 | 66.7 (n = 171) | 66.4 (n = 216) | 0.81 |
| SCCHN | 157 | 60.2 (n = 57) | 61.2 (n = 99) | 0.62 |

MSS, microsatellite stable.

### Early onset uterine leiomyosarcomas

All 68 leiomyosarcoma samples were successfully analysed. The frequencies of the genotypes were similar to the healthy controls (31% wild type (n = 21), 53% heterozygotes (n = 36), and 16% homozygotes (n = 11)). The median age of disease onset was somewhat higher in individuals with wild type (40.9 years, n = 21) than in heterozygous or homozygous SNP309 carriers (39.3 years, n = 47), but this difference did not reach statistical significance (p = 0.24). The possible impact of SNP309 to disease onset was further analysed by comparing the genotype distribution in two subgroups: patients <41 years (n = 33) and in patients ⩾41 years (n = 35) at diagnosis; however, no differences were observed (p = 0.44). There was also no difference observed when the genotype distribution was analysed between local and diffuse tumours (p = 0.21). Finally, the SNP status was analysed according to family history of cancer, but no significant associations were detected between patients with no family history (n = 21) and patients with at least one first degree relative with cancer (n = 36; p = 0.47) (tables 1, 2).

### CRC

Of the 1042 CRC samples, 969 were successfully analysed for SNP309. The frequencies of the genotypes were similar in CRC patients (34% wild type (n = 334), 48% heterozygotes (n = 465), 18% homozygotes (n = 170)) and in healthy controls (p = 0.44). The median age of the CRC patients at diagnosis was 67.8 years in wild type individuals, and 66.8 and 67.5 years in SNP309 heterozygotes and homozygotes, respectively. SNP309 was more frequent in women than in men (p = 0.024). In women, SNP309 was associated with a slightly earlier disease onset (2.7 years, p = 0.026), and patients with microsatellite stable CRC were more frequently homozygous for SNP309 than patients with microsatellite unstable CRC (p = 0.036). Analysis of genotype distribution according to a history of a second cancer at any site (n = 130, including 20 patients with a second CRC) showed a trend towards a slightly higher frequency of homozygous mutations in patients with multiple cancer (24% *v* 16.5% in patients with a single CRC), although this difference did not reach statistical significance (p = 0.096). The frequencies of the genotypes were also analysed according to tumour grade (p = 0.395), Duke's stage (p = 0.235), and family history (no familial cancer *v* one or more cancer in a first degree relative; p = 0.703), but no significant correlations were observed (table 1–2).

### HNSCC

In total, 157 HNSCC samples were successfully analysed for SNP309. In this cohort, 37% (n = 58) of the patients were wild type, 48% (n = 75) heterozygous, and 15% (n = 24) homozygous for SNP309, thus the genotypic distribution was similar to the healthy controls (p = 0.47). The median age of the HNSCC patients at diagnosis was 60.2 years in the group of individuals with wild type alleles, and 60.4 and 63.8 years in heterozygotes and homozygotes, respectively. The genotypes were further analysed between patients ⩽61 years (n = 84) and >61 years (n = 73) at disease diagnosis, but no significant differences in the genotype distribution were observed (p = 0.45). The frequency of SNP309 was also analysed according to tumour size (T1 *v* T⩾2 tumours, p = 0.60), disease progression (p = 0.88), and nodal stage (N0 *v* N+, p = 0.14) but no evidence to support the involvement of SNP309 in any of these variables was obtained (tables 1, 2).

## DISCUSSION

Alterations in *p53* are among the most common genetic changes observed in human malignancies; for instance, in more than 50% of colon adenocarcinomas, *p53* is mutated.[2] In microsatellite unstable CRCs, *p53* mutations occur less frequently, but approximately 50% of these tumours display frameshift mutations in the *BAX* gene, which is involved in the p53 regulated pathway for induction of apoptosis.[11] Alterations in *p53* are also commonly detected in HNSCC; mutations in *p53* have been reported in 40–80% of HNSCCs and inactivation of *p53* through interaction with high risk human papillomavirus in 14–60% of cases.[12–15] The presence of *p53* mutations seems to correlate with worse response to radiotherapy and 5-fluorouracil-cisplatin based chemotherapy in HNSCC, but in both HNSCC and CRC, the prognostic value of p53 remains controversial.[2 16–19 21 20] Adenoviral *p53* gene transfer therapy developed for HNSCC has shown some degrees of success, rendering *p53* a gene of potential clinical relevance in this tumour type.[22] Abnormalities in both *p53* and *MDM2* genes have been reported also in human sarcomas. The *MDM2* gene appears to be amplified in about a third of soft tissue sarcomas, and alterations in p53 have been detected in 17–62% of leiomyosarcomas.[5 23–27] Sample sizes in these studies have been relatively small, and thus no firm conclusion can be drawn.

Single nucleotide polymorphisms in the p53 pathway might explain some of the phenotypic variation in cancer susceptibility. The recent identification of SNP309 in the *MDM2* promoter, resulting in attenuation of the p53 pathway, provides evidence to support this hypothesis.[8] In relatively small sets of hereditary and sporadic soft tissue sarcomas, the frequency of SNP309 was similar to population controls, but it was shown to be associated with earlier tumour formation.[8]

We have examined the role of the *MDM2* promoter SNP309 in three different tumour types; uterine leiomyosarcomas, CRC, and HNSCC, known to be associated with the p53/MDM2 pathway. Overall, the frequency of SNP309 was slightly higher in the Finnish population than that reported previously.[8] We found similar frequencies of SNP309 in all four groups tested: in 68 early onset leiomyosarcomas, 969 CRC samples, 157 HNSCCs, and 185 healthy blood donors.

Analysis of SNP309 genotype distribution according to patients' age at disease onset failed to show a significant

association in the leiomyosarcoma and the HNSCC materials (table 2). Because of limited statistical power, however, subtle changes may have been missed. Bond *et al* reported tumour formation in soft tissue sarcoma occurring 12 years earlier in patients with homozygous SNP309 compared with those without SNP309. Such an association could not be confirmed in our uterine leiomyosarcoma material, although a trend towards a slightly younger age at disease diagnosis was observed in SNP309 carriers. Further studies are needed to confirm the role of SNP309 in other types of sporadic soft tissue sarcomas. Bond *et al* also reported an association of SNP309 with the occurrence of multiple primary tumours in a lifetime in patients with Li-Fraumeni syndrome. None of the leiomoysarcoma patients and only a few HNSCC ($n = 3$) patients had a second tumour, and thus a similar association could not be tested in our materials.

In the population based CRC material, SNP309 was not significantly associated with patient age at diagnosis ($p = 0.17$). Interestingly however, SNP309 was detected in higher frequencies in women than in men ($p = 0.01$, odds ratio 1.4, 95% confidence interval 1.1 to 1.8), and SNP309 in female carriers was linked with an age at diagnosis approximately 2.7 years younger ($p = 0.026$). In addition, a trend towards a higher incidence of homozygous SNP309 in patients with microsatellite stable CRC was observed, which is of some interest, as microsatellite stable and unstable CRCs are different in view of p53 involvement, with microsatellite stable tumours harbouring more *p53* mutations.[11] Furthermore, women with microsatellite stable CRC were diagnosed on average 3.5 years earlier ($p = 0.007$) if they carried SNP309 (tables 1, 2). Because of multiple testing, Bonferroni adjustment was applied and $p < 0.005$ was considered significant. Thus, no statistically significant association between SNP309 and any clinicopathological parameters remained in the CRC material. Interestingly, germline *p53* mutations have been connected with higher cancer risk in female than in male carriers, a difference that cannot be explained by a distortion of sex specific cancer.[28 29] It is therefore tempting to speculate whether a correlation could also exist between germline *MDM2* polymorphisms and sex differences in cancer risk. Subsequent studies are needed to show whether the trends observed in this study hold in other materials.

In conclusion, little evidence was obtained to support the role of SNP309 in cancer predisposition.

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